# Peroxide accumulation without major mitochondrial alteration in replicative senescence

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**Abstract The aim of this work was to determine how ageing changes were related to each other by studying the establishment of a senescent state in cell culture, rabbit articular chondrocytes. A striking increase of the amount of peroxides appeared at 2/3 of the time of cell growth, which is responsible for an oxidative stress, as shown by the appearance of oxidized proteins, the overexpression of *HSP27* gene and the accumulation of HSP27 protein. While no change of the mitochondrial membrane potential was observed all along the cell culture, p21, a protein overproduced in senescent cells, appeared with the peak of peroxides and accumulated.**



*Key words:* Ageing; Oxidative stress; Mitochondrial membrane potential; Chondrocyte

## 1. Introduction

Several theories have been proposed as to the possible causes and mechanisms of ageing. Harman [1] was the first to postulate that mitochondria may play a role in the ageing process. According to this hypothesis ageing would result from the accumulation, during life, of mitochondrial DNA (mtDNA) mutations caused by oxidative damage. Mitochondria appear to be the major source of oxidants which are natural by-products of the respiratory chain activity. mtDNA exposed to free radicals would accumulate detrimental mutations leading to mitochondrial deficiencies and subsequently cellular dysfunction.

Support to this theory have been provided during the last decade. Studies of tissues of young and old individuals show an increase in mtDNA damage (level of 8 oxoguanosine [2]), an increase in the frequency of point mutations like the A3243G mutation [3]. Moreover, using PCR, multiple mtDNA deletions have been detected in various species: rat [4], monkeys [5], and *Caenorhabditis elegans* [6]; usually the frequency remained low, a few percent. From these observations the contribution of mtDNA mutations to ageing seems questionable as the level of deleted molecules reported in human pathological states has to be at least 60% to produce any perceptible phenotype [7].

To investigate further the mitochondrial involvement in the process of ageing, transcription, replicative behavior and enzymatic activities were measured in tissues of young and old individuals. In most studies the steady state level of mitochondrial transcripts was found to decrease with age, in *Drosophila* [8], in rat [9], in brain [10] but not in skeletal muscle of humans [11]. The lower level of transcripts does not seem to reflect a change in mtDNA content, controversial observations were made: an increase [4] or a decrease [12] in rat, a stable amount in *Drosophila* [8].

Again in isolated rat hepatocytes different observations were found regarding the respiratory chain activities. For example a decrease in mitochondrial membrane potential (30%) is associated with an increase in mitochondrial peroxide generation (23%) [13] while in another study the striking increase in hydrogen peroxide induces only minor changes on the mitochondrial membrane potential [14]. Furthermore no measurable increase in the production of hydrogen peroxide was found in the heart of old rats [15]. Numerous reports have shown that a significant decrease of the respiratory chain activity occurs with age but these results have been challenged by recent reports [16].

These various observations do not bring clear evidence of a causal role of mitochondria in ageing. Cellular senescence has been proposed as one possible mechanism contributing to ageing. It certainly cannot be the sole cause of ageing but it may be involved in regenerative tissues and consequently it may have a significant impact at the level of the whole organism. Animal cells with few exceptions have a limited proliferative potential in culture. This property, termed the finite replicative life-span of cells, leads to an irreversible growth arrest termed cellular or replicative senescence. Many studies have demonstrated that the growth potential of cells in culture decreases with increasing the age of donor [17]. In vitro cellular senescence has been known for years, only recently proof of a similar in vivo process was obtained. The most direct evidence for senescent cells in vivo came from a modified histochemical stain for β galactosidase that is specific for senescent fibroblasts in vitro [18].

Facing the sparse and controversial observations we used the in vitro replicative senescence approach to establish the possible correlations between mitochondrial function, peroxide production and specific properties of the senescent state. Rabbit articular chondrocytes were used for two reasons: morphologic changes of in vitro senescent cells are close to changes observed in degenerative articular pathologies found in old people [19]; rabbit mtDNA contains repeated sequences whose generation and maintenance suppose additions, deletions and rearrangements of units [20], furthermore a slight

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*Abbreviations:* $\Delta\Psi$, transmembrane electric potential difference; PBS, phosphate buffered saline; ROS, reactive oxygen species; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine; NAO, nonyl-acridine orange; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; FDA, fluorescein diacetate


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bias in their frequencies was observed in some tissues of old animals [21].

## 2. Materials and methods

### 2.1. Cell culture

Articular chondrocytes were obtained from the shoulder and knee joints of young rabbits (Fauve de Bourgogne), aged 1 month, using an enzymatic release procedure as previously described [22].

In vitro ageing was performed by weekly subculture of cells with a constant seeding density of 4000 cells/cm$^2$ in Dulbecco's modified Eagle's medium/HAM F12 (1:1 in vol.) medium supplemented with ultroser G (Life Technologies) 2%, pyruvate 100 μg/ml and uridine 50 μg/ml.

### 2.2. Northern blot analysis

*2.2.1. RNA isolation.* Extraction of total RNA from confluent cell cultures was performed by the guanidium/caesium chloride method as described elsewhere [23]. The concentrations of RNA were determined by UV absorbance at 260 nm.

*2.2.2. Messenger RNA (mRNA) analysis.* Total RNA (20 μg) was denatured, separated by electrophoresis on a 1.25% agarose gel containing 0.66 M formaldehyde [24], transferred onto a Hybond-N+ membrane (Amersham) and hybridized with $^{32}$P-labeled DNA probes.

### 2.3. Southern blot analysis

*2.3.1. DNA isolation.* Total DNA was extracted from confluent cell cultures. Cells were washed twice with phosphate buffered saline (PBS). DNA extraction was performed using the Puregen kit (Prolabo). DNA concentrations were determined by electrophoresis on a 1% agarose gel in presence of a standard scale of calf thymus DNA.

*2.3.2. Filter hybridization.* 2 μg total cell DNA was digested with *Hin*fI restriction endonuclease, electrophoresed on a 3% agarose gel [20], transferred onto a Hybond-N+ membrane (Amersham) and hybridized with $^{32}$P-labeled DNA probes. Radioactive signals were quantified using a PhosphoImager™ (Molecular dynamics).

### 2.4. Probes for mRNA and DNA analysis

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| *CollII* | A fragment of 405 nucleotides containing the alpha1 (II) gene of rat was obtained by *Eco*RI, *Hin*dIII digestion of the plasmid pMcol2a1-1 [25]. |
| *Hsp27* | Complete rat cDNA |
| *Cytb* | PCR fragment of rabbit mtDNA: nts 327–511 [20]. |
| *CoxII* | PCR fragment of rabbit mtDNA: nts 151–620. |
| *12S* | PCR fragment of rabbit mtDNA: nts 2581–3095. |
| *SRs* | PCR fragment of rabbit mtDNA: nts 1391–1739. |
| *LRs* | PCR fragment of rabbit mtDNA: nts 1791–2481. |
| *18S* | PCR fragment of human DNA: nts 1196–1806. |

### 2.5. Western blot analysis

*2.5.1. Total cellular extract.* Cellular extracts were obtained from confluent cell cultures. Cells were rinsed twice in cold PBS and collected with a scraper, membranes were made soluble by the addition of 0.5% Nonidet-P40. The amount of proteins was determined using the colorimetric method of Bradford [26].

*2.5.2. Tubulin, p21 and Hsp27 immunodetection.* Proteins (20 μg) were separated by SDS–PAGE using a 15% acrylamide 0.2% bisacrylamide gel and transferred onto nitrocellulose membranes (Schleicher and Schuell) according to Towbin et al. [27]. Blots were exposed to the first antibody overnight at 4°C, rinsed in PBS, 0.5% Tween 20 and exposed for one hour, at room temperature, to horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat immunoglobulin serum (Biosystem). The immunoreactivity was revealed using the Amersham ECL kit. The goat polyclonal anti-waf-1 (C-19, Santa Cruz), the mouse polyclonal anti-Hsp27 (SPA-801, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the monoclonal mouse anti-tubulin (AC-40, Sigma Immuno Chemicals) were used in this study.

### 2.6. Oxidized protein detection

Oxidative modifications of proteins by oxygen free radicals were visualized using the Oxidized Protein Detection kit (Oxyblot™, Oncor).

### 2.7. Flow cytometric analysis

*2.7.1. Mitochondrial membrane potential, mitochondrial membrane mass, reactive oxygen species (ROS) production and intracellular esterase activity.* Mitochondrial membrane potential was measured by the retention of 3,3′-dihexyloxacarbocyanine ($DiOC_6(3)$) [28]. Mitochondrial membrane mass was assessed with nonyl-acridine orange (NAO), which binds to all mitochondrial membranes regardless of their membrane potential [29]. The amount of peroxides was measured by cytofluorometry using the dye 2′,7′-dichlorofluorescein diacetate (DCFH-DA). It is deacetylated by intracellular esterases into the non-fluorescent compound DCFH then oxidized to the fluorescent compound DCF by a variety of peroxides, including hydrogen peroxide. [28]. Intracellular esterase activity was measured with fluorescein diacetate (FDA).

Cell stainings were performed as follows: cells were centrifuged and resuspended in complete medium at a concentration of $10^6$ cells/ml. They were then incubated either with $DiOC_6(3)$ (0.1 μM), or DCFH-DA (20 μM), or NAO (5 μM) or FDA (50 μg/ml) for 30 min at 37°C in a humidified 5% $CO_2$:95% air incubator. Prior to flow cytometric analysis, all samples were incubated with propidium iodine (10 μg/ml) for 5 min at 4°C to check cell viability.

*2.7.2. Flow cytometric measurements.* Flow cytometric measurements were performed on a ELITE ESP flow cytometer (Coulter, France). Analyses were performed with $10^4$ cells and data were stored in listmode. Light scatter values were measured on a linear scale of 1024 channels and fluorescence intensities on a logarithmic scale of fluorescence of four decades of log.

## 3. Results

Three experiments were performed using chondrocytes released from three different rabbits. In two experiments cell proliferation has arrested after 10 passages which corresponds to 23 doublings and in one experiment, cell proliferation has arrested after eight passages (17 doublings). The proliferation rhythm had the same profile in the three experiments, it decreased from 1.2 doublings per day at the beginning to 0.07 at the end of the experiment (Fig. 1). We observed, as already described, an increase of the size of cells, an increased number of binuclear/multinuclear cells and an important increase in the density of the cytoskeleton, including actin-containing microfilaments and microtubules [30].

### 3.1. Mitochondrial membrane potential and mitochondrial mass

The measurement of mitochondrial membrane potential (transmembrane electric potential difference, $\Delta\Psi$) is in part representative of the respiratory chain activity. The assessment of $\Delta\Psi$ done by a cytofluorometric method is both qualitative and quantitative. It allows the identification of cell populations with different mitochondrial activities. $\Delta\Psi$ was determined in isolated chondrocytes using the fluorescent dye $DiOC_6(3)$. No measurable change was observed during

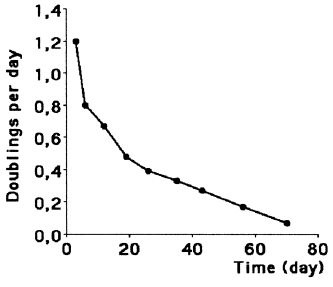


Fig. 1. Proliferation rhythm in a primary culture of rabbit articular chondrocytes.

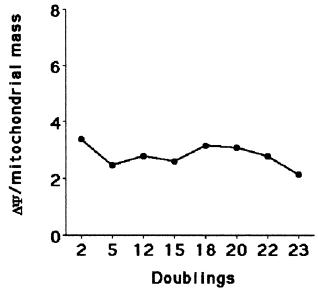


Fig. 2. Ratio of mitochondrial membrane potential ($\Delta\Psi$) to mitochondrial mass in rabbit articular chondrocytes during in vitro ageing. $\Delta\Psi$ and mitochondrial mass were measured by cytofluorometry using $DiOC_6(3)$ and NAO, respectively.

in vitro ageing, a single population of cells was always observed.

Mitochondrial membrane mass was estimated in isolated chondrocytes using NAO, which binds to all mitochondrial membranes regardless of their membrane potential: no significant change was observed through the time of the experiment. So the ratio of mitochondrial membrane potential to mitochondrial mass does not change (Fig. 2).

### 3.2. ROS

The amount of ROS was measured by the production of 2′,7′-dichlorofluorescein (DCF) derived from oxidization of DCFH by intracellular peroxides, previously deacetylated by intracellular esterases. Because DCFH oxidization is inhibited by reduced glutathione (molecules playing a central role in defense against oxidative stress) which amount is pH-dependent [31], this measure does not reflect precisely ROS generation but it is an indirect indicator of the oxidative stress. Intracellular esterase activity was measured with FDA all along the experiment, parallel to the use of DCFH-DA. No change was observed.

Our results (Fig. 3A) showed a clear-cut increase of ROS after 14 doublings, reaching a peak after 18 doublings which corresponds to 2/3 of the cell growth time. In the two other experiments a similar variation of the amount of ROS was observed during the cell culture with a peak at the same time of the cell growth.

### 3.3. Expression of HSP27 gene

The expression of *HSP27* gene was measured by Northern blotting. It is known to be induced by oxidative stress. A slight increase ($\times 1.5$) was observed with a maximum after 20 doublings (Fig. 3B), then the amount of transcripts decreased.

### 3.4. HSP27, p21 immunodetection

The protein HSP27 was revealed with polyclonal antibody. Not detectable at the beginning of the culture it appeared in quantifiable amount after 15 doublings, remained stable from 18 to 23 doublings corresponding to the growth arrest (Fig. 3D). The accumulation of the protein HSP27 and the increase in amount of peroxides were concomitant. The p21 protein, product of the gene waf-1, is known to accumulate during senescence. It appeared after about 18 doublings, which corresponds to the peak of production of peroxides and accumulated up to the end of the culture (Fig. 3D). As the tubulin content was shown to modulate as a function of the protein content and/or the cell volume in ageing chondrocytes it cannot be considered as a very reliable control; staining of the membrane with Ponceau red was used to estimate the gel loading (data not shown).

### 3.5. Oxidized proteins

The amount of oxidized proteins estimated by immunodetection increased until growth arrest; the population of oxidized proteins evolved, oxidization of some polypeptides appeared associated to the peak of ROS, others seemed to accumulate just before the arrest of growth (Fig. 3C).

### 3.6. Mitochondrial DNA

The ratio of mitochondrial DNA to total DNA was determined by Southern blot using *cytb* and *18S* probes. In the experiment presented in Fig. 4A the ratio increased up to 10 fold until 15 doublings then stabilized, in the two other experiments the increase was about three fold; this difference could be correlated to the lower increase in the size of the cells.

The frequencies of long and short repeats were determined by Southern blot analysis using specific probes; an example of the evolution of short repeats is shown in Fig. 4C. No significant modification in the distribution of the frequencies of the various molecular types was observed through the time of the experiment.

### 3.7. mRNA analysis

The expression of three mitochondrial genes was measured by Northern blotting. For each gene studied (*cytb*, *coxII*, *12S*) the evolution was identical: the amount of mRNA increased

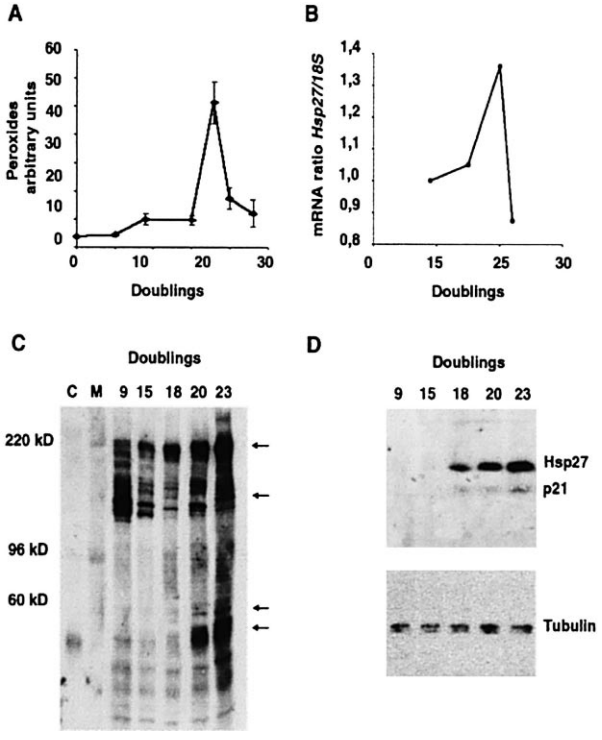


Fig. 3. Establishment of an oxidative stress. A: The intracellular peroxides were measured by cytofluorometry using DCFH-DA. B: The ratio of *Hsp27* mRNA to *18S* RNA was measured by Northern analysis. C: Immunodetection of oxidatively modified proteins after staining of the carbonyl groups introduced into protein side. Lane C: untreated proteins. Lane M: standard treated proteins. D: Immunodetection of Hsp27, p21 and tubulin.

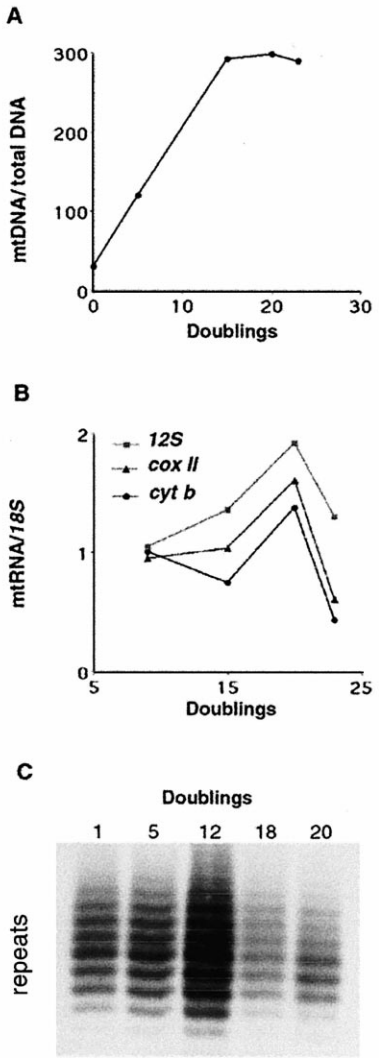


Fig. 4. mtDNA and mtRNA content. A: mtDNA content in rabbit articular chondrocytes during in vitro ageing. The ratio of mtDNA to total DNA was measured by Southern blot analysis using *cytb* and *18S* probes. B: Steady state levels of mitochondrial transcripts, *coxII*, *cytb* mRNAs and ribosomal *12S* RNA expressed as a ratio to the nuclear *18S* RNA. C: Distribution frequencies of short repeats. Total DNA was digested by *Hin*fI endonuclease and the repeats were revealed by Southern blot hybridization using a specific probe.

1.5 fold until 16 or 17 doublings then decreased (Fig. 4B). In all cases the peak corresponds to the oxidative stress as shown by the expression of *HSP27* and the production of ROS.

As already known the expression of collagen II, specific of articular chondrocytes, dropped down during the first divisions and remained at a very low level during the whole the experiment (results not shown).

## 4. Discussion

Comparison of cells of young and old individuals has brought a lot of data, sometimes controversial, about characteristic ageing changes. The aim of this study was to try to determine how changes were related to each other; this was made possible by the observation of the establishment of a senescent state in cell culture, rabbit articular chondrocytes in this case. While the proliferative capacity decreases (Fig. 1) the mean size of the cells, the number of multinuclear cells, the density of the cytoskeleton increase, all these changes have already been described as characteristics of ageing cells. The main result of this work is the observation of a critical period at 2/3 of the life-span of the culture, whatever the number of population doublings before the arrest of growth (17 or 23 depending on the experiment). At this time, a peak of peroxides was observed which is responsible for an oxidative stress as judged by the appearance of oxidized proteins (Fig. 3C), the overexpression of *Hsp27*, a gene known to be induced by a stress (Fig. 3B) and the accumulation of Hsp27 protein (Fig. 3D). At the same time the p21 protein, an inhibitor of the cyclin-dependent kinase, overproduced in senescent cells [32], became measurable and increased up to the end of the culture. If mitochondria play an important role in ageing via the production of ROS we would expect some alterations of mitochondrial functions. No major corresponding change in the mitochondrial membrane potential was found neither in the mitochondrial mass estimated by the fixation of NAO to the inner membrane. As NAO binds to the cardiolipin one must admit that its amount does not change during this period. Citrate synthase activity was used by several authors as an estimate of the mitochondrial mass; we measured this activity in one experiment, it remained stable (data not shown). From these results an important dysfunction of the respiratory chain, correlated to a burst of ROS, can be ruled out.

The mitochondrial mRNAs *cytb* and *coxII* increase ($\times 1.5$) up to the peak of production of ROS then decrease to a lower level ($\times 0.5$) which agrees with the observation made in tissues of old individuals as compared to young ones. The decrease to a lesser extent of the mt rRNA 12S results possibly from a better stability. There is no correlation between the evolution of the amount of mt mRNA and that of mtDNA; the increase in mRNA took place always at 2/3 of the cell culture and at the same level ($\times 1.5$) in the three experiments while the increase in mtDNA was observed at the beginning of the cell culture and was different from one experiment to another (3–10 fold).

The distribution pattern of the frequencies of short and long repeat remains stable all along the cell culture. A slight bias towards longer molecular types was found in ovaries or testis of old animals [21]. These observations and those presented in the present work indicate that the slight differences in the frequencies of the long repeats in old animal are specific of a tissue rather than of ageing itself.

We conclude that during the establishment of a senescent state in cell culture there is a clear-cut peak of ROS which appears correlated with a marked increase in proteins involved in the response to a stress (HSP27) and in senescence (p21) without dramatic alteration of the mitochondrial functions.

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